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Characterization of cDNA for nodulin-75 of soybean: A gene product involved in early stages of root nodule development

(*Rhizobium*/nodule-like structures/proline-rich proteins/DNA sequence)

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ABSTRACT **Establishment of a nitrogen-fixing root nodule is accompanied by a developmentally regulated expression of nodulin genes, only some of which, the so-called early nodulin genes, are expressed in stages preceding actual nitrogen fixation. We have isolated soybean cDNA clones representing early nodulin genes and have studied clone pENOD2 in detail. The cDNA insert of this clone hybridizes to nodule-specific RNA of 1200 nucleotides in length. The RNA that was hybrid-selected by the cloned ENOD2 DNA was *in vitro* translated to produce two nodulins with an apparent M_r of 75,000, the N-75 nodulins. These two nodulins differ slightly in charge and one does not contain methionine. The amino acid sequence deduced from the DNA sequence shows that proline accounts for 45% of the 240 residues in these nodulins and the sequence contains at least 20 repeating heptapeptide units. The amino acid composition of none of the (hydroxy)proline-rich (glyco)proteins described in plants resembles the composition of the N-75 nodulins, especially with respect to the high glutamic acid and the low serine content. This suggests that the N-75 nodulins belong to a hitherto unidentified class of presumably structural proteins. The genes encoding the N-75 nodulins were found to be expressed in nodule-like structures devoid of intracellular bacteria and infection threads, indicating that these nodulins do not function in the infection process but more likely function in nodule morphogenesis.**

The formation of nitrogen-fixing nodules on the roots of leguminous plants induced by bacteria of the genera *Rhizobium* and *Bradyrhizobium* involves the specific expression of a number of plant genes called nodulin genes (1–3). In a description of nodule development, Vincent (4) distinguishes between three stages in nodule development denoted as "preinfection", "infection and nodule formation", and "nodule function". In the preinfection stage, the *Rhizobium* bacteria recognize their host plants and attach to the root hairs, an event that is followed by root hair curling. At the moment, nothing is known about specific plant genes that are involved in this stage. In the next stage, the bacteria enter the roots by infection threads while concomitantly the dedifferentiation of some cortical cells results in the formation of meristems. The infection threads grow toward the meristematic cells; bacteria are released into the cytoplasm of about half of these cells and develop into bacteroids. In the final stage, further differentiation of nodule cells occurs leading up to a nitrogen-fixing nodule. Most studies on the expression of nodulin genes so far have been confined to the final stage of root nodule development. But the steps involved in root nodule formation show that major decisions determining the development of a root nodule are made in the stages preceding the establishment of a nitrogen-fixing nodule. We have shown (5) that nodulin genes are differentially expressed during development and that in pea at least two nodulin genes are transcribed in the second stage of root nodule formation. These genes are referred to as early nodulin genes. Here we report the detailed analysis of a soybean cDNA clone representing an early nodulin gene.

MATERIALS AND METHODS

Growth Conditions for Plants and Bacteria. Soybean plants [*Glycine max* (L) Merr. cv. Williams] were cultured as described for pea plants (6) but at 28°C. At the time of sowing the soybean seeds were inoculated with *Bradyrhizobium japonicum* USDA110 or *Rhizobium fredii* USDA257. Both strains were cultured as described (7).

Isolation of Nodules. Nodules were excised from the roots with a scalpel. For samples prior to 6 days after sowing and inoculation, a 4-cm root segment of the upper part of the main root (where root nodules normally would develop) was harvested. Nodules were frozen in liquid nitrogen and stored at −70°C until use.

Isolation of Nucleic Acids. Total RNA from nodules and roots was isolated as described (2) and poly(A)$^+$ RNA was obtained by oligo(dT)-cellulose chromatography (8). Plasmid DNA was isolated by the alkaline lysis method (8).

Construction of cDNA Library. DNA complementary to poly(A)$^+$ RNA isolated from nodules from 21-day-old plants was synthesized with reverse transcriptase (Anglian Biotechnology, Essex, England) and second strand synthesis was performed under standard conditions (8). The double-stranded cDNA was treated with S1 nuclease and size-fractionated on a sucrose gradient. Double-stranded cDNA with a length of 500 base pairs (bp) or more was tailed with dC and then annealed to *Pst* I-cut oligo(dG)-tailed pBR322 (Boehringer Mannheim) in a 1:1 molar ratio. The hybridized mixture was used to transform *Escherichia coli* RR1 (8). On the average, 5000 transformants were obtained per μg of poly(A)$^+$ RNA.

Differential Screening of the cDNA Library. Individual transformants were picked, transferred to 96-well microtiter plates containing LB medium, 15% glycerol, and 12.5 μg of tetracycline per ml, and grown for 16 hr at 37°C. Two replica filters were made on GeneScreen*Plus* (New England Nuclear). After 16 hr of bacterial growth on LB agar containing 12.5 μg of tetracycline per ml, the filters were prepared for hybridization according to the GeneScreen*Plus* manufacturer's manual. Probes for differential screening were prepared from poly(A)$^+$ RNA isolated from segments of 5-day-old, uninfected roots and from nodules 10 and 21 days after inoculation, as in the construction of the cDNA library except that 10 μCi of [^{32}P]dATP (specific activity = 3200 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear) was used.

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Abbreviations: 2D, two-dimensional; ORF, open reading frame.

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The filters were hybridized for 72 hr at 65°C to either root or nodule ^{32}P-labeled cDNA in 0.9 M NaCl/90 mM sodium citrate/5× Denhardt's solution (8)/10 mM EDTA/0.5% $NaDodSO_4$/100 μg of sonicated, denatured calf thymus DNA per ml/20 μg of poly(A) per ml. The filters were washed twice in 0.3 M NaCl/30 mM sodium citrate/0.1% $NaDodSO_4$ for 15 min at room temperature and twice in 75 mM NaCl/7.5 mM sodium citrate/0:1% $NaDodSO_4$ for 30 min at 65°C.

***In Vitro* Translation of Total RNA.** Total RNA (3 μg) from roots or nodules was translated *in vitro* in a wheat germ extract (Bethesda Research Laboratories) in a 15-μl mixture to which 15–30 μCi of [^{35}S]methionine or 6 μCi of [^{3}H]leucine was added (according to the manufacturer's manual). Translation products were separated by two-dimensional (2D) gel electrophoresis followed by fluorography of the dried gel to preflashed Kodak XAR5 film (2).

Hybrid-Released Translation. For hybrid-released translation, the pENOD2 insert (10–15 μg of DNA) was denatured and applied to 0.5-cm^2 discs of diazophenylthioether-paper (Bio-Rad) essentially as described (8). Total soybean RNA from 16-day-old nodules (750 μg) was then hybridized to the filter-bound DNA in 300 μl of 50% (vol/vol) deionized formamide/0.1% $NaDodSO_4$/0.6 M NaCl/4 mM EDTA/80 mM Tris·HCl, pH 7.8. Hybridization was initiated at 40°C and the temperature was slowly decreased to 37°C over a period of 6 hr. After washing, the bound RNA was eluted (8) and dissolved in 3 μl of H_2O; 1.5 μl was translated and analyzed as above.

RNA Transfer Blot Analysis. Total soybean RNA was denatured in dimethyl sulfoxide/glyoxal, electrophoresed in 0.8% agarose gels (8), and transferred to GeneScreen (New England Nuclear) as described (2). The blots were prehybridized for 6 hr in 50% (vol/vol) deionized formamide/1 M NaCl/0.05 M Tris·HCl, pH 7.5/5× Denhardt's solution/0.1% $NaDodSO_4$/100 μg of denatured salmon sperm DNA per ml and hybridized with nick-translated (8) probes. Hybridization was performed for 16 hr at 42°C. Blots were washed twice for 15 min at 42°C in 0.3 M NaCl/30 mM sodium citrate/0.1% $NaDodSO_4$ and twice for 30 min at 42°C in 75 mM NaCl/7.5 mM sodium citrate/0.1% $NaDodSO_4$.

DNA Sequencing. Standard techniques were used for cloning into M13 and pUC vectors (9) and for dideoxy (10, 11) and for Maxam–Gilbert (12) sequencing. The DNA sequence data were stored and analyzed with programs written by R. Staden (13) on a microVAX/VMS computer.

Cytology. Nodules were fixed for 16 hr in 3% glutaraldehyde in 50 mM sodium phosphate buffer (pH 7.2). After fixation the nodules were rinsed with the same buffer, dehydrated in a graded ethanol series, embedded in Technovit 7100 (Kulzer, Wehrheim, F.R.G.), cut into 5-μm sections, stained with toluidine blue, and examined under a light microscope.

RESULTS

Characterization of pENOD2. Out of a cDNA library prepared against poly(A)$^+$ RNA of soybean root nodules, 10 cDNA clones were isolated that specifically hybridized with the nodule cDNA probe transcribed from 10-day-old plant poly(A)$^+$ RNA. These clones represent early nodulin genes and will be designated pENOD to distinguish them from pNOD clones that represent nodulin genes expressed at later stages of development. Cross-hybridization studies revealed 8 clones with common sequences, of which the clone pENOD2 with an insert length of 1000 bp was chosen for further characterization.

On DNA transfer blots of *Eco*RI-digested soybean genomic DNA, five restriction fragments were found to hybridize with ^{32}P-labeled pENOD2, whereas no hybridization was observed with *B. japonicum* DNA (not shown). Hence, the cloned ENOD2 DNA is encoded by the soybean genome and its gene is probably part of a small gene family.

Clone pENOD2 hybridized to a mRNA of 1200 nucleotides; the concentration of the ENOD2 mRNA is highest at day 10 and decreases during further nodule development (Fig. 1*A*). Thus, the ENOD2 gene is apparently transiently expressed during soybean nodule development, although in some experiments the concentration of ENOD2 mRNA remained nearly constant between 10 and 21 days.

For comparison of the expression of the ENOD2 genes with that of nodulin genes expressed later in development, a leghemoglobin (Lb) cDNA clone (pLb), selected from the cDNA library by hybridization with a soybean Lb cDNA clone (14) made available by K. Marcker (University of Aarhus, Denmark), was used. The difference in time of expression between ENOD and Lb genes is illustrated in Fig. 1*B*. The RNA transfer blot was first hybridized with pENOD2 and was subsequently probed with pLb. The Lb mRNAs start to appear when the ENOD2 mRNA concentration is already decreasing.

pENOD2 Codes for N-75 Nodulin. To identify the early nodulin encoded by pENOD2, mRNA was hybrid-selected by pENOD2 and translated *in vitro* in the presence of [^{35}S]methionine. The results showed that the pENOD2-encoded polypeptide has an apparent M_r of 75,000 with an isoelectric point around 6.5 (Fig. 2*C*). In accordance with the nomenclature established for nodulins (15), the identified polypeptide is named N-75. After *in vitro* translation of the pENOD2-selected RNA in the presence of [^{3}H]leucine, two polypeptides were found, one of which comigrated with the polypeptide detected after translation with [^{35}S]methionine whereas the other, more prominent, polypeptide was slightly more basic (Fig. 2*B*). The two polypeptides obtained from hybrid-released translation should be closely related and are each encoded by a different member of the ENOD2 gene family. The polypeptides, both referred to as N-75, were also easily detected as nodulins in the 2D pattern of the polypeptides obtained upon *in vitro* translation of total nodule RNA (Fig. 2*A*).

The translation products of these mRNAs of 1200 nucleotides in length have an apparent M_r of 75,000, whereas mRNA of that length has a coding capacity for a polypeptide of, at most, M_r 45,000. This notable discrepancy prompted us

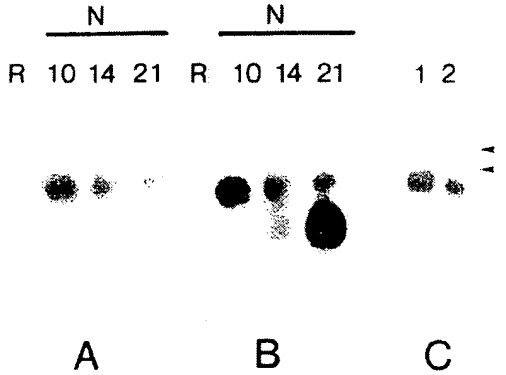


FIG. 1. Autoradiographs of two identical RNA transfer blots (*A* and *B*) containing 15 μg of total RNA isolated from 5-day-old uninoculated roots (R) and nodules (N) harvested 10, 14, and 21 days after sowing and inoculation with *B. japonicum* USDA110. (*C*) Autoradiograph of an RNA transfer blot containing 15 μg of RNA isolated from nodules harvested 14 days after sowing and inoculation with *B. japonicum* USDA110 (lane 1) and from nodule-like structures collected 4 weeks after inoculation with *R. fredii* USDA257 (lane 2). The blots were hybridized with ^{32}P-labeled pENOD2 (*A* and *C*) and with pENOD2 and pLb in consecutive order (*B*). The positions of the rRNAs are indicated by arrowheads.

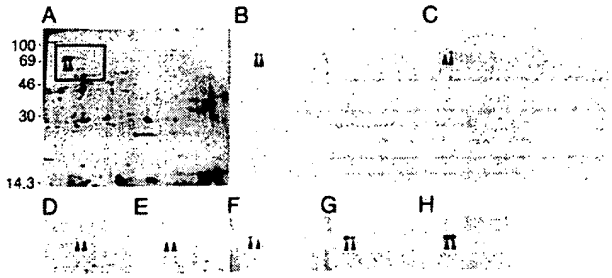


FIG. 2. Characterization of the early nodulin cDNA clone pENOD2 by hybrid-released translation (*A–C*) and time-course analysis of the expression of the nodulin-75 genes during nodule development (*D–H*). Total RNA from 16-day-old soybean nodules (*A*) and RNA eluted from filter-bound pENOD2 DNA (*B* and *C*) were translated in a wheat germ extract in the presence of [^{3}H]leucine (*A* and *B*) or [^{35}S]methionine (*C*). The products obtained were separated by 2D gel electrophoresis and fluorographed. The positions of the N-75 nodulins are indicated by arrowheads. In *D–H* only the region of the 2D gel within the square indicated in *A* is shown that represents the [^{3}H]leucine *in vitro* translation products obtained from RNA of 5-day-old, uninfected soybean root (*D*), from root segments of infected soybean plants 6 days after sowing and inoculation with *B. japonicum* USDA110 (*E*), and from soybean nodules 7 (*F*), 10 (*G*), and 13 (*H*) days after sowing and inoculation with the same strain. In *A* the molecular weight of the markers is indicated as $M_r \times 10^{-3}$.

to sequence the cDNA insert of pENOD2 to see if the deduced amino acid sequence could explain the peculiar physical properties of the encoded polypeptides. At the same time information on the structure may provide clues on a possible function of the N-75 nodulins. The sequencing data (Fig. 3) reveal that the *Pst* I insert of the pENOD2 cDNA clone contains 998 nucleotides including a short 3′ poly(A) tail but excluding the dCdG nucleotides generated in the cloning procedure. At most 200 nucleotides of the 5′ end of the 1200-nucleotide-long mRNA, including the initiation codon and coding sequences for the N terminus of the polypeptide, are thus missing in this clone. Two open reading frames (ORFs) occur in the same strand (Fig. 3). From one ORF, designated ORF-1, 728 nucleotides are found in the cDNA clone (positions 13–741; Fig. 3), and this ORF ends with two successive termination codons. A second ORF, designated ORF-2, not in phase with the first one, comprises 611 nucleotides (positions 14–625; Fig. 3) and ends in a single termination codon. Both ORFs are followed by 3′ nontranslated regions of about 250 (ORF-1) and 375 (ORF-2) nucleotides, respectively, in which two potential poly(A) addition signals are present (Fig. 3).

Although both ORFs seem to be able to code for a nodulin of about the same size, several lines of evidence indicate that only ORF-1 corresponds to a nodulin N-75. First, ORF-1 gives rise to a completely different polypeptide (241 amino acids of which none is methionine) than ORF-2 (203 amino acids of which 20 are methionine). Such entirely different polypeptides will differ in physical properties. The two N-75 *in vitro* translation products will therefore most likely be related polypeptides with a similar amino acid sequence, derived from two different mRNAs, and not from two ORFs of one mRNA. If then one of the N-75 *in vitro* translation products is shown to contain no methionine (Fig. 2*C*) the other N-75 nodulin may have a low methionine content. But the ORF-2-derived polypeptide contains 20 methionines, indicating that it is rich in methionine. We therefore deduce that ORF-1 encodes a nodulin-75. This conclusion is supported by the absence of methionine in the amino acid sequence deduced from this ORF.

Moreover, though both ORFs encode a polypeptide containing repeating peptide sequences, the repetitive amino acid sequences occurring in the polypeptide encoded by ORF-1 are better preserved than those present in the ORF-2-derived polypeptide. This indicates that an evolutionary tendency exists for a functional conservation of the polypeptide coded for by ORF-1. We therefore propose that ORF-1 will exclusively be used for the generation of a nodulin N-75.

ORF-1 shows that the N-75 nodulins are peculiar proline-rich proteins. N-75 contains a repetitive sequence of 10 or 11 amino acids that is repeated at least 20 times. Embedded in this repetitive sequence a heptapeptide sequence is found that is conserved in 19 of 20 units (Fig. 3). This repeated heptapeptide sequence is Pro-Pro-Xaa-Glu-Lys-Pro-Pro, in which 17 times Xaa = histidine and 3 times Xaa = tyrosine or leucine. Three or four amino acids that are not as conserved, mainly proline, glutamic acid/glutamine, and tyrosine, flank the heptapeptide repetitive sequence. Two apparent partials of the heptapeptide repeat are found at positions 133 and 451 within the sequence. Neither α-helix nor β-sheet conformations were found using the method of Lim (16). The high proline content of the N-75 nodulins probably explains the discrepancy between the observed apparent M_r of 75,000 and the coding capacity of a 1200-nucleotide mRNA. A similar aberrant migration behavior on $NaDodSO_4$/polyacrylamide gels is found for the proline-rich protein collagen (17).

N-75 Is Involved in Nodule Morphogenesis. To form an idea of the process in which the proline-rich N-75 protein might be involved, we attempted to correlate the beginning of expression of the N-75 genes with a defined stage in root nodule formation. Total RNA was isolated from tap root segments of 6-day-old inoculated plants, where nodules are not yet visible, and from nodules harvested 7, 10, and 13 days after sowing and inoculation. RNA preparations were analyzed by *in vitro* translation using [^{3}H]leucine followed by 2D gel electrophoresis. The area of the 2D gel where N-75 nodulins are found is shown in Fig. 2 *D–H*. Both N-75 proteins first appear at day 7 (Fig. 2*F*) and then increase in concentration up to day 13 (Fig. 2 *G* and *H*).

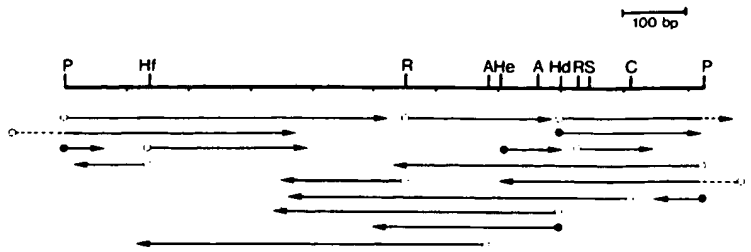
By examining the nodule structures formed by *R. fredii* USDA257, it proved possible to distinguish between the infection process and the differentiation into a nodule structure. On commercial soybean cultivars, this fast-growing *Rhizobium* strain cannot form nitrogen-fixing root nodules but forms nodule-like structures that are not able to fix nitrogen.

Histological examination of these nodule-like structures revealed that they arise from a combination of cell swellings and randomly oriented cortical cell divisions (Fig. 4). In these nodule-like structures, no infected plant cells or infection threads were observed and none of the structures examined had an organization with vascular bundles at the periphery, similar to normal nodules. Apparently the formation of such a nodule-like structure does not require an infection process. By transfer blot analysis, N-75 RNA could be detected in RNA isolated from these nodule-like structures (Fig. 1*C*).

DISCUSSION

A cDNA library from soybean root nodules has been analyzed for copies of mRNA transcripts of early nodulin genes. These genes are expressed in the early stage of root nodule development when the nodule structure is being formed. One of the clones, pENOD2, was characterized in detail. The nucleotide sequence of the *Pst* I insert of pENOD2 has been determined and the derived amino acid sequence shows that 45% of the amino acids of N-75 is proline and that the amino acid sequence is organized in highly repetitive units (Fig. 3).

In an effort to derive a function for N-75 in root nodule development from these sequence data, we have surveyed the occurrence of proline-rich proteins in plants and their

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ORF-1        P H E K T P P E Y L P P P H E K P P P E Y L P P H E K P P P E Y Q P P H
GGGGGGGGGGGACCCCATGAAAAAACACCACCTGAGTATCTACCTCCTCCTCATGAGAAACCACCACCAGAATACCTACCTCCTCATGAGAAACCGCCACCAGAATACCAACCTCCTCAT   120
ORF-2         P M K K H H L S I Y L L L M R N H H Q N T Y L L M R N R H Q N T N L L M

E K P P H E N P P P E H Q P P H E K P P E H Q P P H E K P P P E Y E P P H E K P
GAGAAACCACCCCATGAGAATCCACCACCGGAGCACCAACCACCTCATGAGAAGCCACCAGAGCACCAACCACCTCATGAGAAGCCACCACCAGAGTATGAACCACCTCATGAGAAACCA   240
 R N H P M R I H H R S T N H L M R S H Q S T N H L M R S H H Q S M N H L M R N H

P P E Y Q P P H E K P P P E Y Q P P H E K P P P E Y Q P P H E K P P P E H Q P P
CCACCAGAATACCAACCACCTCATGAGAAGCCACCACCAGAATACCAACCACCTCATGAGAAACCACCACCAGAATACCAACCACCTCATGAGAAGCCACCACCAGAGCACCAACCACCT   360
 H Q N T N H L M R S H H Q N T N H L M R N H H Q N T N H L M R S H H Q S T N H L

H E K P P E H Q P P H E K P P P E Y Q P P H E K P P P E Y Q P P Q E K P P H E K
CATGAGAAGCCACCAGAGCACCAGCCACCTCATGAGAAGCCACCACCAGAGTATCAACCACCTCATGAGAAACCACCACCAGAATACCAACCTCCTCAAGAAAAGCCACCACATGAAAAA   480
 M R S H Q S T S H L M R S H H Q S I N H L M R N H H Q N T N L L K K S H H M K N

P P P E Y Q P P H E K P P P E H Q P P H E K P P P V Y P P P Y E K P P P V Y E P
CCACCGCCAGAATACCAACCTCCTCATGAAAAGCCACCACCAGAACACCAACCTCCCCATGAAAAGCCACCACCAGTGTACCCACCCCCTTATGAGAAACCACCACCAGTGTATGAACCC   600
 H R Q N T N L L M K S H H Q N T N L P L K S H H Q C T H P L M R N H H Q C M N P

P Y E K P P P V V Y P P P H E K P P I Y E P P P L E K P P V Y N P P P Y G R Y P
CCTTATGAGAAGCCACCCCCAGTAGTGTATCCACCTCCTCATGAGAAACCACCCATTTATGAGCCACCGCCATTGGAGAAGCCAACCGGTCTACAATCCCCCACCTTATGGCCGCTATCCA   720
 L M R S H P Q *

 P S K K N * *
CCATCCAAGAAAAACTAATAACCACTTGCCTGCGTCACATGTTTTGGTCTACTCAAACTTAGACCTGCCCTTTGTCATATAAAGCTTTTTGTTTCTGTTTAAGATCTCAAGTACAATATG   840

TCCCTTCTGCATGCACTACTTCTTCAAAATAAAGGCTTTATGCCTATGTATAATACTCTACTTTAATTCTCCTTTCACCATCGATATTGTAATGTCAACTACTAGTGTGGGTTTATCTAT   960
                    +++++
GGCTATAATAAGTTTTTCTTTGTGTTTAAAAAAAAAAAAAAAAAAAAAAAACCCCCCCCCCCCCCCCCCCTG
     +++++
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FIG. 3. Partial restriction map, sequencing strategy, and nucleotide sequence of the *Pst* I fragment from pENOD2. Sequencing was performed by the Maxam–Gilbert method (12) (open circles) and by the dideoxy method of Sanger (10, 11) (closed circles). The arrows depict the direction and extent of sequencing from the indicated site. The dashed lines correspond to sequences originating within the vectors. In the DNA sequence, nucleotides are numbered on the right of the sequence. The predicted amino acid sequence is shown in standard single-letter code for both ORFs and the characteristic heptapeptide repeat is overlined in ORF-1. The two partial repeats are indicated by a dotted line. Termination codons (*) and potential poly(A) addition sites (+++) are also marked. P, *Pst* I; Hf, *Hin*fI; R, *Rsa* I; A, *Acc* I; He, *Hae* III; Hd, *Hin*dIII; S, *Sph* I; C, *Cla* I.

assumed functions. All proline-rich proteins that have been described in plants so far contain hydroxyproline that is posttranslationally formed and in most cases subsequently glycosylated. Although it remains to be established whether the soybean N-75 proteins become hydroxylated and glycosylated *in vivo*, soybean nodule tissue has been described as extremely hydroxyproline-rich (18). Also the apparent absence of a class of proline-rich proteins in plants justifies the assumption that the nodulins N-75 belong to one of the classes of hydroxyproline-rich glycoproteins. There are four major classes of hydroxyproline-rich glycoproteins in plants (19): (*i*) the cell wall structural hydroxyproline-rich glycoproteins or extensins (20, 21), (*ii*) the arabinogalactan proteins (22), (*iii*) the solanaceae lectins (23), and (*iv*) hydroxyproline-rich agglutinins (24).

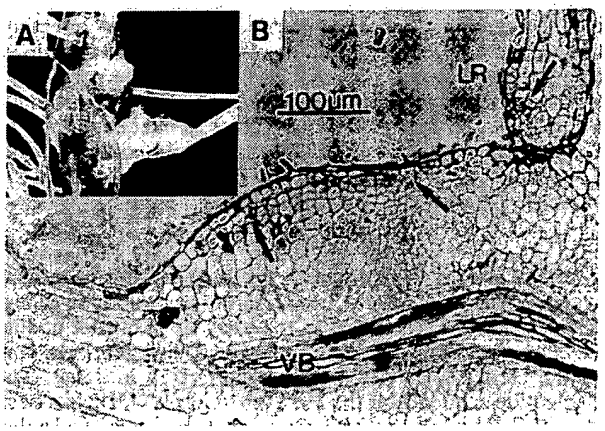

FIG. 4. (*A*) Nodule-like structure on soybean roots obtained 4 weeks after inoculation with *R. fredii* USDA257. (*B*) Part of a longitudinal section of a nodule-like structure. Cell divisions in the outer and inner cortical cell layers are indicated by arrows. VB, root vascular bundle; LR, lateral root. (Bar = 100 µm.)

Extensins are associated with the cell walls of most dicotyledonous plants (25). They are assumed to play a role in maintaining the integrity of the primary cell wall (26) and they may be important in controlling growth and development. Extensins have been shown to accumulate in plant cell walls upon wounding (27) and pathogen attack (28, 29) in what is considered a defense response. The amino acid sequences of extensins are characterized by the occurrence of a repeating Ser-Hyp-Hyp-Hyp-Hyp pentapeptide. Neither this pentapeptide nor the typical high serine content is found in the amino acid sequence of N-75 (Fig. 3). The nodulins N-75 are therefore not closely related to extensins.

After comparison of the amino acid composition of representatives of each of the classes mentioned above with N-75, it can be concluded that the amino acid composition of none of the (hydroxy)proline-rich (glyco)proteins analyzed so far resembles the amino acid composition derived for N-75. Nor is N-75 homologous to the hydroxyproline-rich (glyco)proteins P33 (30) and "protein 4" (31), recently identified, using the same criteria as above. We therefore may be dealing with a hitherto unknown class of (hydroxy)proline-rich proteins, characterized by a remarkably low content of serine and a surprisingly high content of glutamic acid. Both the highly repetitive nature of the amino acid sequence and the high proline content suggest that the N-75 nodulins are structural proteins.

To gain a better understanding of the biological function of the N-75 nodulins in root nodule development, we have studied the expression of their genes as a function of time (Fig. 2 *D–H*). The expression of the N-75 genes is first detectable at 7 days after sowing and inoculation, when the nodule meristems just emerge through the root epidermis. We were not able to detect N-75 RNA in 7-day-old tap root pieces with visible nodule structures, whereas we could detect N-75 mRNA in excised nodule structures of the same age. Therefore it cannot be excluded that these genes are already expressed earlier than 7 days after sowing and inoculation but in fewer cells and at a similar or lower level than at 7 days.

Irrespective of the possible expression of these genes before 7 days, a strong stimulation of N-75 gene expression occurs from 7 to 13 days. The N-75 proteins should therefore be involved in a developmental event that proceeds during this period. Around day 7 in our growth system the nodules emerged through the root epidermis. This stage of development equals stage VII as described by Calvert *et al.* (32), and cytological observations have shown that in this stage the meristems start to differentiate into nodule structures (32). Some of the meristem cells have been invaded by infection threads from which the rhizobia are beginning to be released. To examine whether the induction or stimulation of the expression of the N-75 genes is specifically related to the infection process, on the one hand, or the formation of a nodule structure, on the other, we have looked for N-75 gene expression in nodule-like structures formed by *R. fredii* USDA257. This strain induces the formation of nodule structures devoid of intracellular bacteria and infection threads (Fig. 4), in which, however, early nodulin N-75 RNA is detectable (Fig. 1*C*). The expression of the N-75 genes in nodules without bacteria or infection threads strongly suggests that N-75 is not involved in the infection process, but more likely in nodule morphogenesis.

Quantitative light microscopic observations on nodule initiation have shown (32) that up to the stage in which the meristems emerge through the epidermis, development can stop. However, when a meristem has reached the "emergence stage" it will continue to develop into a mature nodule. The stimulation of the expression of the N-75 genes coincides therefore with the moment the soybean nodule meristems have reached an apparently critical developmental stage. The expression of the N-75 genes might therefore reflect the definitive "commitment" of the meristems to develop into a nodule. The nature of the involvement of the hydroxyproline-rich nodulins N-75 in this commitment remains to be established.

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Stable Transformation of Soybean Callus by DNA-Coated Gold Particles

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ABSTRACT

Immature soybean (*Glycine max* L.) embryos from commercially important cultivars were the targets of rapidly accelerated, DNA-coated, gold particles. Protoplasts were prepared from these tissues and propagated in culture under selection conditions for the introduced neomycin phosphotransferase II gene. Kanamycin-resistant calli were obtained at a rate of approximately 10^{-5}. Enzyme assays and Southern blot hybridization confirmed the expression of the foreign gene and its stable integration into the soybean genome. Our results show that particle acceleration can be used for the introduction of foreign DNA into the soybean genome and indicate the technique may be useful in the recovery of engineered plants by transformation of regenerable tissues.

Successful application of genetic engineering procedures to soybean has been limited by the inability to regenerate plants from transformed tissues. Successful use of nononcogenic *Agrobacterium* Ti vectors has not been reported, although oncogenic transformation of soybean by virulent *Agrobacterium* strains and the axenic culture of excised tumors on hormone-free media has been achieved (7, 15, 16, 22, 23). Various assays and markers have been used to confirm transformation events, but caution should be exercised in drawing conclusions regarding putative transformation events (3). We have recently described efficient transformation of soybean protoplasts by electroporation and the recovery of stably transformed calli and roots (4). This technique, however, will not be useful in the recovery of genetically engineered soybean plants until regeneration from protoplasts is accomplished. Klein *et al.* (13) used DNA-coated tungsten microprojectiles to obtain transient expression of foreign DNA in intact epidermal cells of *Allium cepa* (onion). However, these authors did not demonstrate stable transformation of the plant cells. Activity through transient expression does not necessarily indicate that stable transformants will be obtained. We have used a microparticle technique to deliver foreign DNA into intact soybean embryo cells and have recovered stable transformants using a protoplast selection regimen (4). Enzyme assays and Southern blot hybridization confirm the integration of the foreign gene and its expression in the soybean genome. Results from these experiments indicate that particle acceleration can lead to stable transformation at rates comparable to electroporation. The properties of the transformants obtained by these two methods also appear similar.

MATERIALS AND METHODS

Vector Construction. Plasmid pCMC1022 is a pBR322-derived vector that includes a NPT II[1] coding region under the control of the cauliflower mosaic virus (CaMV) 35s promoter and the nopaline synthase (nos) polyadenylation region. The plasmid was derived from pCMC1021 (4) by substituting a 35s promoter fragment (nucleotide position 6654 to position 7436 of the published sequence [9]) for the nos promoter. The promoter fragment had previously been modified by addition of synthetic *Xho*I and *Hin*dIII linkers to the ends of the fragment upstream and downstream, respectively, of the cap site (10). A restriction map of pCMC1022 is shown in Figure 1A. Plasmids were prepared from amplified cultures by the method of Ish-Horowicz and Burke (11), purified by isopycnic centrifugation in CsCl-ethidium bromide gradients (6), and chromatographed on NACS-52 resin (BRL, Bethesda, MD).

Preparation of DNA-Coated Particles. Seventy μg of pCMC1022 (1 mg/ml in distilled water) was used to suspend 3.5 mg of 1 to 5 μm gold spheres (Alfa Chemical Co). The suspension was then dried under a stream of nitrogen and resuspended in 100% ethanol to a final concentration of 2 mg beads/ml.

Particle Delivery. Of the gold bead/DNA suspension, 162 μl was pipetted onto the surface of an 18 mm square of aluminized foil and allowed to settle undisturbed. The carrier sheet was then air-dried and placed on top of an electrical, arc-discharge gun. The gun was comprised of a short polyvinyl chloride pipe (13 mm i.d.) with electrodes mounted approximately 5 mm below the upper end. Power was provided by a 25 kV, 2 μF capacitor that was charged by a regulated, 24 kV DC power supply. The spark gap was bridged by a droplet of water before each discharge. The carrier sheet was supported on a 15 mm expansion spacer. A 100 mesh screen was suspended above the gun assembly to catch the carrier sheet, allowing the accelerated gold spheres to continue through the screen and into the target. The target was comprised of an array of embryos mounted on a 1% water-agar Petri dish inverted over the retaining screen. The entire gun/target assembly was evacuated to 500 mm of mercury before discharging the capacitor. A more detailed description of the arc-discharge gun will be published elsewhere.

Protoplast Isolation. Four- to eight-mm zygotic soybean (*Glycine max* L.) embryos were excised from greenhouse-grown plants (cultivars Williams 82, Mandarin Ottawa, and Hardin), were chopped and plasmolyzed directly after treatment with microparticles, and were incubated in the enzyme mixture described by Lu *et al.* (14) for 4 to 5 h at room temperature on a gyrotary shaker at 20 rpm. Protoplasts were released by sieving the digestion mixture through a 54 μm stainless steel screen and rinsing with 2 ml of a 9% mannitol/salt solution (8). The filtrate was transferred to 15 ml conical tubes and was centrifuged at 135*g* for 8 min. The supernatant was discarded, and the protoplasts were washed twice in the same solution by resuspension and centrifugation. Aliquots of the protoplast suspension were diluted, stained with fluorescein diacetate and counted on a hemocytometer grid under UV illumination. The number of protoplasts containing one or more microparticles was also de-

[1] Abbreviation: NPT II, neomycin phosphotransferase II.

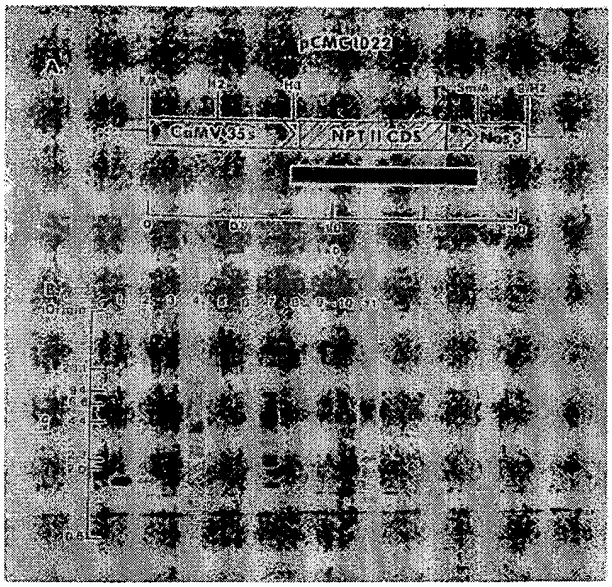

FIG. 1. A, Restriction map of the chimeric NPT II gene from pCMC1022. The blocks indicate the CaMV 35s promoter fragment (35s), a fragment from Tn5 which includes the NPT II coding sequence (NPT II CDS) (1) and a fragment from pTiT37 which includes the nopaline synthase polyadenylation signals (nos 3'). The region corresponding to the NPT II coding sequence is indicated by cross-hatching. The arrow indicates the direction of transcription. The thin lines represent vector sequences derived from pBR322, most of which is not shown. The thickened line indicates the region included in the hybridization probe used in panel B. The location of pertinent restriction sites is shown. B, Southern blot analysis of transformed soybean calli. DNA prepared from independently derived, kanamycin-resistant calli was digested with Hinc II and analyzed by Southern blot hybridization as described in "Materials and Methods." Samples in each lane are as follows: lane 1, 10 μg of DNA from nontransformed soybean callus plus 33 pg of pCMC1022; lane 2, as lane 1 but with 6.6 pg pCMC1022; lane 3, 10 μg of DNA from nontransformed soybean callus; lanes 4 to 11, 10 μg of DNA from several kanamycin-resistant calli. Lanes 1 and 2 correspond to five copies and one copy, respectively, of pCMC1022 per soybean genome based on a genome size of 6×10^9 base pairs (2). Lanes 7 and 8 correspond to calli derived from electroporation. The remaining lanes correspond to calli derived from microsphere treatment. The position of molecular length markers (*Hin*dIII digest of λ-DNA) is indicated on the left. Autoradiographic exposure was for 72 h.

termined. Protoplasts were then diluted with protoplast media to the desired density for plating (2×10^4 per ml).

Protoplast Culture and Selection. Protoplasts were cultured in Corning 75 cm^2 tissue culture flasks containing 10 ml of Kao's medium (12) as described elsewhere (4). Kanamycin-resistant colonies appeared 2 to 3 weeks following plating on solid media. Cultures were amplified on the same media containing kanamycin at 50 and 100 mg/L.

NPT II Assays. Callus extracts were prepared as described by Platt and Yang (17) and assayed for NPT II activity by the procedure of Reiss *et al.* (18). The amount of enzyme present in each sample was estimated by comparison to a known amount of purified enzyme included on the same gel.

Southern Blot Hybridization. DNA was prepared from lyophilized tissue by the method of Taylor and Powell (21). DNA was digested with restriction endonucleases under the conditions recommended by the supplier (New England Biolabs). The fragments were resolved by electrophoresis on an 0.8% agarose gel and then transferred to nylon membranes (Biodyne membranes, Pall, Irvine, CA) as described by Southern (20). ^{32}P-Labeled RNA hybridization probes were synthesized *in vitro* using an SP6 transcription system (Promega Biotec, Madison, WI) and α-^{32}P GTP (300 Ci/mmol, Amersham; 1 Ci = 37 GBq). The template for probe synthesis produces a runoff transcript corresponding to the minus strand of the coding sequence for NPT II. Reaction conditions recommended by the supplier, using 100 μCi of radiolabeled GTP, were used. Incorporation was typically 60 to 80%. Hybridization and washing conditions were as described by Church and Gilbert (5). Filters were analyzed by autoradiography using X-Omat AR5 film (Kodak) at −80°C with two intensifying screens (Cronex Lightning plus, Du Pont).

RESULTS

Five to seven immature embryos were arranged on a 60 mm plate containing 5 ml of 1% water-agar and subjected to particle acceleration using an arc discharge gun as described in "Materials and Methods." Approximately 0.1 mg of particles per square centimeter of the carrier sheet was found to be a good median loading rate. This density of particle application resulted in good survival of the treated tissues, excellent protoplast recovery following digestion of the bombarded embryos, and uniform penetration of cells by the accelerated particles. Our experiments showed that acceleration and penetration of the cells by the particles could vary with the tissue being treated. The number of carrier particles and the arc discharge energy could be adjusted to obtain uniform coverage of the target tissues. Under the conditions described in "Materials and Methods," a significant percentage of the carrier particles arrive at the target with the correct velocity to penetrate the cells on the target surface without destroying an unacceptable proportion of them. Effective penetration was limited to a depth of 5 to 7 cell layers. This portion of the process afforded a population in which 0.1 to 5% of the cells contained at least one particle.

Treated embryos were chopped and plasmolyzed, and protoplasts were isolated as previously described (4). Protoplast yields were somewhat lower from treated tissues than from nontreated tissues, presumably due to a reduction in the number of viable cells in the original explant as a result of injury. Microscopic examination of the isolated protoplasts revealed that approximately 10^{-3} protoplasts contained one or more gold particles. Following isolation, the protoplasts were plated as described elsewhere (4). Kanamycin selection was applied on low-melting-point agarose media 3 weeks following the original plating in Corning 75 cm^2 flasks. Kanamycin-resistant colonies appeared 2 to 3 weeks following this plating and continued to appear for an additional 6 to 8 weeks (Fig. 2). Calli were transferred to fresh media containing kanamycin when they weighed 30 mg. The majority of the transferred colonies proliferated rapidly. Tissues were amplified for further analyses on the same basal media supplemented with 100 mg/L kanamycin.

Enzyme assays demonstrated that all of the kanamycin-resistant colonies (about 100) exhibited NPT II activity (Fig. 3). A comparison of lanes 2 to 7 shows relative differences in enzyme activity which likely reflects different levels of gene expression. Several callus lines that exhibited different levels of NPT II activity were plated on a range of kanamycin concentrations and their fresh weights determined at time intervals. Callus line E1, derived from electroporation experiments, was also included in this experiment for comparison. The results are shown in Figure 4. Although the level of enzyme specific activity varied more than fivefold among the calli, their level of resistance to kanamycin appears similar, at least up to kanamycin concentrations of 400 mg/L. The calli exhibiting the highest levels of NPT II activity (Nos. 2, 4, 5) appear to show slightly higher resistance to kanamycin; however, the observed differences may not be significant.

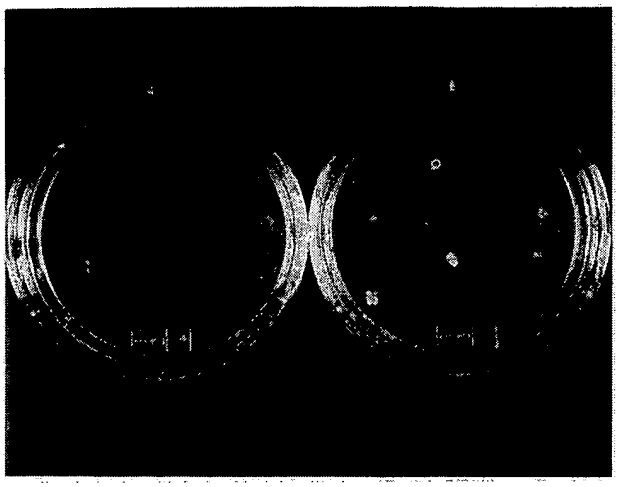

FIG. 2. Selection of calli for resistance to kanamycin. Transformed calli were obtained only when tissues were treated with gold particles coated with pCMC1022 (right). Plating on nonselective medium resulted in the recovery of colonies with a plating efficiency of about 1% in both cases.

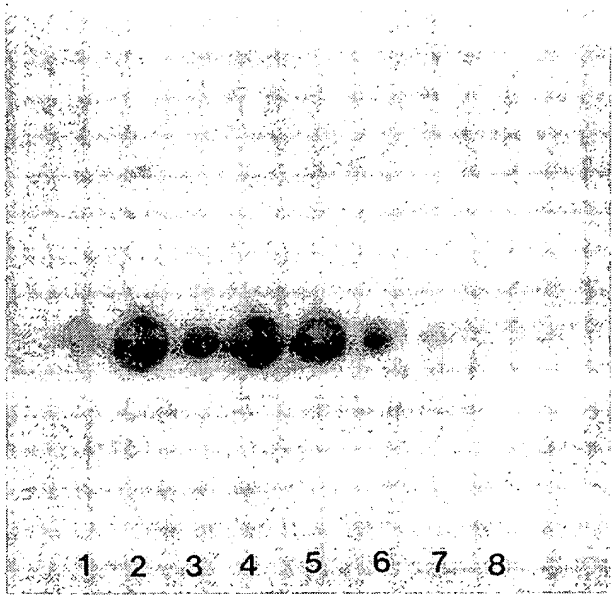

FIG. 3. NPT II activity in kanamycin-resistant calli: callus extracts were assayed for NPT II activity as described in "Materials and Methods." Autoradiographic exposure was for 2 h. Samples applied to each lane are as follows: lane 1, purified NPT II enzyme; lanes 2 to 6, kanamycin-resistant soybean callus resulting from particle acceleration. Lane 7, transformed soybean callus derived from electroporation. Lane 8, non-transformed, protoplast-derived soybean callus.

DNAs prepared from kanamycin-resistant calli were analyzed by Southern blots (Fig. 1B) to determine the fate of the introduced DNA. The organization of NPT II sequences is complex, a result similar to that previously observed for transformants obtained from electroporation (4) and other transformation techniques employing naked DNA (19). The results are consistent with random integration of the transforming DNA into the soybean genome. The number and pattern of hybridizing fragments varies dramatically from one callus to another. When

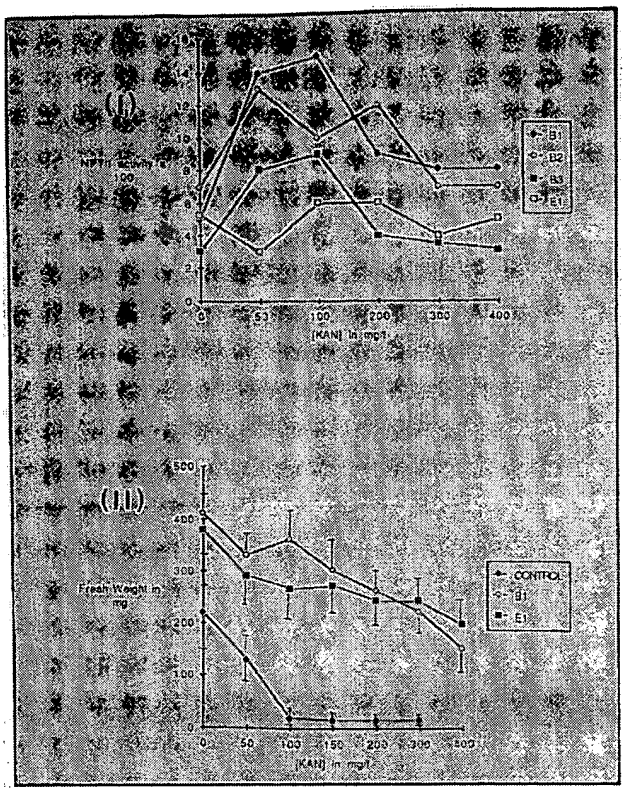

FIG. 4. NPT II activity (I) and fresh weight (II) *versus* [kanamycin] (in mg/L) of transformed lines derived from particle acceleration (B) and electroporation (E). Activity is expressed as ng of enzyme per 20 μg of protein. Vertical bars indicate standard error.

digested with the restriction endonucleases *Ava*I and *Hin*dIII (Fig. 1A), the DNA from each callus can be shown to contain at least one copy of the intact NPT II coding region (data not shown). This result is consistent with the kanamycin-resistant phenotype and the presence of NPT II enzyme activity in these calli. When digested with *Hin*cII, as shown in Figure 1B, DNAs from several calli exhibit the expected fragment (*e.g.* lanes 1, 2, 4, 5, 7, 8, 10, and 11), but DNAs from calli Nos. B6 and B7 (lanes 6 and 9, respectively) produced only fragments larger than expected. This indicated that a recombination event had occurred within the expected *Hin*cII fragment. The latter fragments presumably include a break-point of the recombination with soybean DNA and must also include active genes since they are the only fragments containing NPT II sequences in these calli. In addition to the expected fragment, novel restriction fragments (*i.e.* fragments not predicted by the plasmid restriction map), are detected. These fragments presumably result from rearrangement and/or modification of the input DNA.

DISCUSSION

The objective of these experiments was to evaluate the potential of particle acceleration as an alternative for the introduction of foreign DNA into plant cells. *Agrobacterium*, direct DNA transfer (PEG/calcium phosphate or electroporation), and microinjection, although useful for some plants, are not generally useful because of various barriers. These barriers include limited host range, low efficiency, or difficulty in regenerating plants from the transformed tissues that are produced. The use of microparticles as DNA carriers (13) might overcome these obstacles. Since the technique appears to be adaptable to a wide

variety of plant tissues, large numbers of cells can be treated simultaneously and it is applicable to intact, regenerable tissues. We have used a soybean protoplast system as a model since we were previously able to efficiently recover stably transformed soybean cultures by this method (4). Admittedly, passage of the treated cells through a protoplast phase negates an important potential advantage of the microparticle technique. However, our results demonstrate that introduction of DNA into intact plant cells by means of high velocity microparticles can result in stable transformation. Thus, there is no intrinsic feature of the microparticle-carrier technique that precludes stable integration and expression of foreign DNA.

The general features of DNA integration and gene copy number were found to be similar in tissues derived from either electroporation or particle acceleration. This suggests that although the delivery of DNA by these two methods is different, the same pathways of recombination and integration are operating on the DNA once it is delivered into the cell. Both the number of gene copies and the level of NPT II activity varied widely from one callus to another; however, no correlation between copy number and level of expression was observed. Many of the NPT II gene fragments detected on Southern blots appeared rearranged and, thus, may not be competent for expression of the NPT II protein. The observation that callus No. B6, which contains only a single copy of the chimeric NPT II gene, is among the calli showing the highest levels of NPT II activity suggests that the flanking soybean DNA is influencing expression of this gene. The levels of kanamycin-resistance observed among the transformed lines do not vary widely despite relatively large differences in the levels of NPT II activity. This indicates that even a relatively low level of NPT II expression confers a high degree of resistance.

Our results indicate it may be possible to use particle acceleration for the delivery of foreign DNA into competent or regenerable tissue with the subsequent recovery of plants via organogenesis or somatic embryogenesis. The selection procedure we employed probably produces clonal cell cultures which can be recovered despite transformation rates on the order of 10^{-5}. In contrast, plants derived from treatment of intact tissues are likely to be chimeras of both transformed and nontransformed cells, which will complicate the identification or selection of rare transformation events. Recovery of transformants among self-pollinated progeny of treated plants may be effective if transformation of the germ line can be achieved at practical rates.

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DNAssist Version 1.03 ©2000 Hugh Patterton
Alignment 0

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Sequence 1   1   TTTGATCTTTATACTTGTATTTTTTTTTTGTTTTAGTTTCTATACTTAAAAATTCTGTTTTA  62

Sequence 0   59  TTATTTTTACGCCTTAGTTTTCTAGCAATCTAAAACTGATATAAAATAGAAGTATAACGACT  120
Sequence 1   63  TTATTTTTACGCCTTAGTTTTCTAGCAATCTAAAACTGATATAAAATAGAAGTATAACGACT  124

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Sequence 1   373 CTCTATCACACTTGTTATTTTCTCACTATTTTTTTCTCTTGTTTCTCTGTTATTTTCACTCT  434

Sequence 0   431 AAAACTGGAGTAATATGTTTATGACTACAACACATTTTGACATGACTTAGGATTAACATATA  492
Sequence 1   435 AAAACTGGAGTAATATGTTTATGACTACAACACATTTTGACATGACTTAGGATTAACATATA  496

Sequence 0   493 TTATGATAAAATAACTAAAGATTG 516
Sequence 1   497 TTATGATAAAATAACTAAAG    516
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